Table 1. Steady state kinetics of Tth and *Tsp.* AK16D ligase [a]

Ligase	K_m (nM)	k_{cat} (min^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
Tth	87	56	1.1×10^7
*Tsp.*AK16D	104	38	0.62×10^7

[a]: Results represent the average of at least three experiments.

The steady state properties of *Tsp.* AK16D ligase were similar to *Tth* ligase, indicating that the catalytic channels are highly conserved in *Thermus* ligases. The average *K*m value of about 90 nM for *Thermus* ligases is similar to the *K*m value of 50 nM for *E. coli* ligase (Modrich, et al., J Biol Chem, 248(21):7495-7501 (1973), which is hereby incorporated by reference) and about 10-fold higher than vaccinia virus ATP-dependent ligase (Sekiguchi, et al., Nucleic Acids Res, 25(4):727-734 (1997), which is hereby incorporated by reference). The average *k*cat value of about 45 turnovers per min for *Thermus* ligases is higher than the *k*cat value of 28 turnovers per min for *E. coli* ligase (Modrich, et al., J Biol Chem, 248(21):7495-7501 (1973), which is hereby incorporated by reference).

Example 12 - Ligation Of Gapped Or Inserted DNA Duplex Substrates

Gapped substrates were formed by deleting one or two nt from the 3' hydroxyl site of oligonucleotide LP3'C, and inserted substrates were formed by adding one or two nt at the 3' hydroxyl site of oligonucleotide LP3'C. Gapped or inserted duplexed DNA sequences are distinctively different from normal nicked substrate. Under our experimental conditions, no ligation was detectable with 1-nt (i.e. nucleotide) or 2-nt gapped or 2-nt insertion substrates for either *Tth* or *Tsp.* AK16D ligase (Figure 7A). As for 1-nt insertion substrates, only A insertion gave a trace amount of ligated products for both ligases (Figure 7A). All other 1-nt insertions at the ligation junction could not be ligated. In contrast, Hin ligase and *Chlorella* ligase demonstrate observable ligation with 1-nt gap (Ho, et al., J Virol, 71(3):1931-1937 (1997) and Cheng, et al., Nucleic Acids Res, 25(7):1369-1374 (1997), which are hereby incorporated by reference). In the case of vaccinia ligase, the ligation of 1-nt gap is negligible but the formation of DNA-adenylate intermediate is significant, suggesting the major impact of using 1-nt gapped substrate is on nick

closure (Shuman, S., Biochemistry, 34(49):16138-16147 (1995), which is hereby incorporated by reference). The formation of DNA-adenylate intermediate with the *Thermus* enzymes was not observed, suggesting that most of the gapped or inserted substrates may have abolished the possibility of completing the second step in the ligation cycle — adenylation of DNA substrate at the 5' phosphate. The 1-nt A insertion mis-ligation could be due to slippage (Figure 7B). Although *Thermus* ligase slippage is far less than *Thermus* DNA polymerase, it does occur at a low frequency. Given the fact that the adjacent nt is a T, the slippage could have occurred at 5' phosphate side where a 5'A/C mismatch is ligated (Luo, et al., Nucleic Acids Res, 24(15):3071-3078 (1996), which is hereby incorporated by reference). It is unlikely that the enzyme tolerates slippage on the 3' side , because a 1 nt C insertion did not yield detectable ligation product (Figure 7).

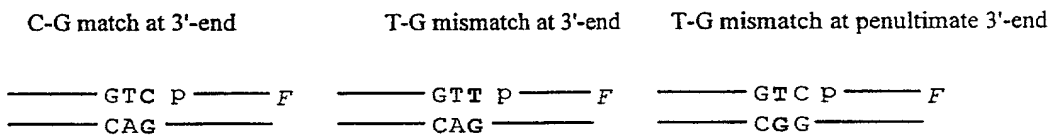
Example 13 - *Thermus* DNA Ligase Fidelity

Tth DNA ligase is more discriminative when the mismatch is located at the 3' side of the nick junction. 3'G/T or 3'T/G is the only mismatch that shows observable mismatch ligation (Luo, et al., Nucleic Acids Res, 24(15):3071-3078 (1996), which is hereby incorporated by reference). To evaluate the fidelity of the cloned *Tsp.* AK16D ligase, the rate ratio of match over 3'T/G mismatch ligation was compared with wild-type and K294R mutant *Tth* DNA ligases along with T4 ligase from a commercial source (Table 2).

Table 2. DNA ligase fidelity [a]

Ligase	Enzyme Concentration (nM)	Initial rates of C-G match (fmol/min)	Initial rates of T-G mismatch at 3'-end (fmol/min)	Initial rates of T-G mismatch at penultimate 3'-end (fmol/min)	Ligation fidelity 1[b]	Ligation fidelity 2[c]
T4	0.5	1.4×10^{2}	2.8	7.1	5.0×10^{1}	1.9×10^{1}
Tth -wt	1.25	5.5×10^{1}	6.5×10^{-2}	2.9×10^{-1}	8.4×10^{2}	1.9×10^{2}
Tth-K294R	12.5	1.5×10^{2}	2.3×10^{-2}	4.3×10^{-1}	6.3×10^{3}	3.4×10^{2}
Tsp. AK16D	12.5	1.3×10^{2}	2.5×10^{-2}	1.2×10^{-1}	5.1×10^{3}	1.1×10^{3}

[a] The reaction mixture consisted of 12.5 nM nicked DNA duplex substrates, indicated the amount of DNA ligases in ligation reaction buffer. T4 DNA ligase fidelity was assayed at 37 °C, thermophilic ligase fidelity was assayed at 65°C. Five µl Aliquots from a 160 µl reaction mixture were removed at 0, 10, 20, 30, 40, 50, 60 s for reactions containing matched substrates and at 0, 1, 2, 3, 4, 5, 6 h for reactions containing mismatched substrates, and mixed with 5 µl of stop solution. Samples (5 µl) were electophoresed through an 8 M urea-10% polyacrylamide gel as described. Fluorescently labeled ligation products were analyzed and quantified using Genescan 672 version 2.0 software (Applied Biosystems, Foster City, CA). The results were plotted using DeltaGraph Pro3 software (DeltaPoint Inc., Monterey, CA). The initial rates were determined as the slope of linear range of the gragh with the x-axis as the time and the y-axis as the amount of the ligation product generated. A schematic illustration of matched and mismatched substrates are as follows:



[b] Ligation fidelity 1= Initial Rate of C-G match / Initial Rate of T-G mismatch at 3'-end.

[c] Ligation fidelity 2= Initial Rate of C-G match / Initial Rate of T-G mismatch at penultimate 3'-end. The concentrations of DNA ligases used in each experiment are as indicated. Results were calculated as the average of at least two experiments.

T4 ligase demonstrated high catalytic efficiency toward both match and 3'T/G mismatch substrate such that a ligation fidelity of 50 was obtained. *Thermus* ligases appeared to be less efficient in match ligation as evidenced by the requirement of higher enzyme concentration to achieve comparable match ligation rate. However, under the same assay conditions, *Thermus* enzymes were far less prone to ligate a 3'T/G mismatch. As a result, the fidelity of *Thermus* enzymes was 17- to 126-fold higher than T4 ligase (Table 2, Ligation fidelity 1). The fidelity of the newly cloned *Tsp.* AK16D ligase was similar to K294R *Tth* mutant but 6-fold higher than wild-type *Tth* enzyme. A DNA-adenylate intermediate was observed with 3'T/G mismatch